Effects of Solubilization on the Inhibition of the P-Type ATPase from Maize Roots by *N*-(Ethoxycarbonyl)-2-Ethoxy-1,2-Dihydroquinoline

David K. Brauer*, Mary Gurriel, and Shu-I Tu

Plant and Soil Biophysics Research Unit, Eastern Regional Research Center, Agricultural Research Service, United States Department of Agriculture, 600 East Mermaid Lane, Room 1118, Philadelphia, Pennsylvania 19118

ABSTRACT

The biochemical events utilized by transport proteins to convert the chemical energy from the hydrolysis of ATP into an electrochemical gradient are poorly understood. The inhibition of the plasma membrane ATPase from corn (*Zea mays* L.) roots by *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline (EEDQ) was compared to that of ATPase solubilized with *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate (3-14) to provide insight into the minimal functional unit. The chromatographic behavior of the 3-14-solubilized ATPase activity during size exclusion chromatography and glycerol gradient centrifugation indicated that the solubilized enzyme was in a monomeric form. Both plasma membrane-bound and solubilized ATPase were inhibited by EEDQ in a time- and concentration-dependent manner consistent with a first-order reaction. When the log of the reciprocal of the half-time for inhibition was plotted as a function of the log of the EEDQ concentration, straight lines were obtained with slopes of approximately 0.5 and 1.0 for membrane-bound and 3-14-solubilized ATPase, respectively, indicating a change in the number of polypeptides per functional ATPase complex induced by solubilization with 3-14.

Enzymes that link the transport of ions to either the hydrolysis or synthesis of ATP have been classified into three broad categories of transport ATPase, designated as P-, V-, and F-type (28). These ATPases can be distinguished by their biochemical, physical, and physiological properties. The F-type ATPase couples the flux of ions, usually protons, down their electrochemical gradient to the synthesis of ATP. The other two classes of ATPases couple the transport of ions against their electrochemical gradient to the release of free energy from ATP hydrolysis.

The mechanism by which the energy from ATP is converted into the movement of ions by the V- and P-type ATPases is poorly understood. A generalized reaction scheme has been proposed for the P-type ATPases in which transport reactions are tightly coupled to the reactions, leading to phosphorylation and dephosphorylation of the enzyme (24, 28). Soon after the discovery of the V-type ATPase, it was apparent that a reaction mechanism in which ATP hydrolysis and ion transport were tightly linked could not account for the differential inhibition and activation of the activities of V-type ATPase (32). Thus, an indirect coupling hypothesis was proposed for V-type ATPases (32). According to an indirect coupling mechanism, the ATPase is composed of three functional domains. One leads to ATP hydrolysis, another to ion transport, and the third domain links the other two. Recent evidence indicates that the coupling between ATP hydrolysis and ion transport by P-type ATPases may not be as rigid as initially envisioned (9, 16, 21–23), and a reaction mechanism based on indirect coupling may account for certain data better than a direct coupling mechanism.

An understanding of the subunit composition of the P-type ATPase is necessary to advance our understanding of the molecular steps leading to ATP hydrolysis and ion transport. It is quite apparent that one subunit of the P-type ATPase, designated as α, is responsible for ATP binding, formation of the phosphorylated enzyme intermediate, and binding of the transport ions (11, 28). However, several enzymes within this class of ATPase, including the (Na + K) and (H + K) ATPase, have at least a second subunit, designated as β (13, 24, 27). In the (Na + K) ATPase, the β subunit is responsible for binding a certain class of inhibitor (24), and as such is believed to play a role in enzyme regulation. Recently, the occurrence of a γ subunit that may have a role in ion transport has been proposed (15), although its importance to transport has been questioned (19). Most researchers still regard the α subunit as the site of catalytic functions leading to both transport and ATP hydrolysis.

The minimal number of α subunits in the smallest functional unit of P-type ATPases has been an area of intense study. It has been proposed from irradiation inactivation studies that two α subunits acting in a reciprocal manner constituted the smallest functional unit of the P-type ATPase in native membranes (7, 12, 22). However, this is not the only structural unit that P-type ATPases can attain. Solubilized P-type ATPases capable of sustained rates of ATP hydrolysis can be either monomers (12, 20, 21, 24) or aggregates of three or more α subunits (14, 17, 24, 31, 33), depending on the type of detergent, the ratio of detergent to protein, and the temperature used in solubilization. Recently, experiments have demonstrated that monomers of the α subunit also can catalyze ion transport (18, 20). The ability of monomers of the α subunit to catalyze transport and the lack of a membrane-bound system to calibrate irradiation inactivation studies in the past have led some researchers to question the validity of the model that dimers of the α subunit are the smallest functional unit in membranes (18). In addi-

temperature over 3 min, and then 4 μL of various concentrations of EEDQ in methanol were added. After incubation at 18 to 22°C for 0 to 90 min, 5-μl aliquots were diluted to a final volume of 100 μL for the assay of ATPase activity. Samples treated with EEDQ and immediately assayed for enzymic activity had rates of ATP hydrolysis within 95% of samples treated with an equal concentration of methanol (data not shown). Samples incubated with an equivalent volume of solvent at 18 to 22°C had rates of ATP hydrolysis comparable to untreated controls (data not shown).

Where indicated, the EEDQ treatment was terminated by diluting the vesicles 5- to 10-fold with ice-cold 20 mM Hepes resuspension buffer. The vesicles were collected after centrifuging at 55,000 rpm in a TLA-100 rotor for 60 min at 4°C. The vesicles were then suspended in 20 mM Hepes resuspension buffer.

Electrophoresis

SDS-PAGE was performed using the Phast gel system from Pharmacia with 7.5% (w/w) acrylamide gels. Samples were diluted with an equal volume of 2.5% (w/v) SDS in 20 mM Hepes (pH 8.0) immediately before electrophoresis.

RESULTS AND DISCUSSION

Characterization of 3–14-Solubilized ATPase

Treating corn root plasma membranes with 0.75 mg of 3–14 per mg of protein solubilized virtually all of the vanadate-sensitive ATPase activity and over 90% of the total protein (data not shown). The approximate molecular mass of the solubilized ATPase was initially characterized by glycerol density centrifugation. When the 3–14-solubilized proteins were subjected to glycerol gradient centrifugation in the presence of 3–14, most of the protein as well as ATPase activity equilibrated as one peak just within the glycerol gradient (Fig. 1). Therefore, the relative density of the ATPase-detergent complex was not much different from that of the other proteins from plasma membranes. Because the ATPase did not migrate rapidly through the glycerol gradient, the 3–14-solubilized enzyme could not be in a high molecular mass complex, as observed with fungal and plant ATPase solubilized with other detergents like lyso-PC (11, 14, 17, 25, 30). Rather, the 3–14-solubilized enzyme was likely composed of one or a few 100-kD subunits, as indicated previously by irradiation inactivation (12).

The addition of a detergent with a relatively high critical micelle concentration (i.e. DOC or octylglucoside) has been used to remove a vast majority of the detergent initially used to solubilize a membrane-bound enzyme (14, 17). Under these conditions, P-type ATPases quite often are isolated as high molecular mass aggregates by density centrifugation (2, 11, 14, 17, 25). In our hands, treatment of 3–14-solubilized ATPase with DOC did not alter the profile of ATPase activity and protein after glycerol gradient centrifugation in the presence of DOC with or without 3–14 (data not shown).

The 3–14-solubilized ATPase treated with DOC was subjected to HPLC to estimate more accurately its molecular mass (Fig. 2). The ATPase eluted from the column as a discrete peak with a retention that was slightly greater than the vast majority of the solubilized protein as determined by A_{280}. The elution volume for vanadate-sensitive ATP hydrolysis was less than that of BSA (66 kD) and similar to that observed for the cross-linked BSA dimer (132 kD). This elution profile is consistent with the hypothesis that the ATPase is a monomer and was supported by a calibration curve constructed from the elution of soluble proteins of known molecular masses in the presence of 3–14 and DOC. Thus, the results from both glycerol gradient centrifugation and HPLC indicate that the 3–14-solubilized ATPase is in a monomeric form. These results support the conclusions concerning subunit composition from irradiation inactivation studies (12).

Characteristics of EEDQ Inhibition

When plasma membranes were incubated with various concentrations of EEDQ, there was a time-dependent decrease in ATP hydrolysis (data not shown). Inhibition appeared to be irreversible, because membranes washed free of EEDQ before assaying had reduced rates of ATPase activity (data not shown). The concentration dependence of vanadate inhibition of ATP hydrolysis catalyzed by the P-type ATPase in plasma membrane vesicles was not affected when 50% of the initial ATPase activity was inhibited by EEDQ treatment (data not shown).

The kinetics of ATP utilization by the plasma membrane ATPase were significantly altered by EEDQ treatment. The maximum velocity of the vanadate-sensitive ATP hydrolysis by plasma membranes treated with 2 mM EEDQ for 15 min at 18 to 22°C was approximately 55% less than that of untreated vesicles, whereas the K_m for ATP in the presence

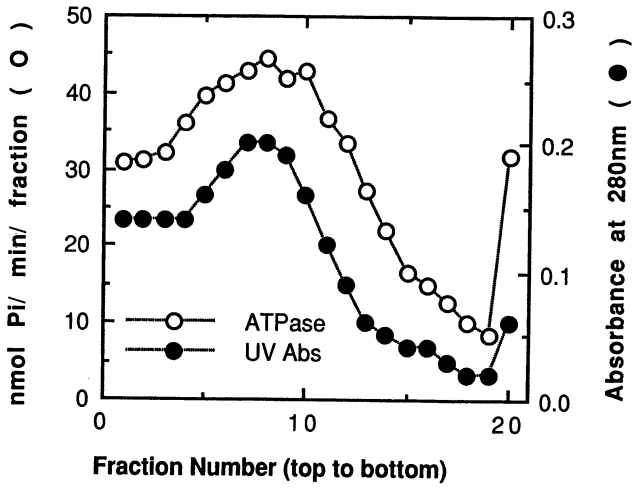

Figure 1. Distribution of 3–14-solubilized plasma membrane protein and vanadate-sensitive ATPase activity after glycerol gradient centrifugation. Plasma membranes were treated with the detergent 3–14, and the solubilized portion was layered over a 15 to 40% (w/v) glycerol gradient as described in "Materials and Methods." After centrifuging for a minimum of 15 h, the gradient was fractionated into 0.65-mL aliquots. The aliquots were then assayed for protein by absorbance at 280 nm (●) and for vanadate-sensitive ATPase activity (O).

tion, the target size of gramicidin is dependent on the lipid composition of the membranes (3).

We have approached the determination of the apparent functional unit of the corn root P-type ATPase by comparing the inhibition induced by EEDQ[1] in both membrane and solubilized preparations. EEDQ is able to catalyze the formation of peptide bonds within and between polypeptides by activating carboxylic acids and allowing them to react with nearby amines (4, 5). As such, EEDQ has been found to be a potent inhibitor of other P-type ATPases (1). Data from irradiation inactivation of the red beet storage tissue P-type ATPase were consistent with the hypothesis that the smallest functional units in native membranes and membranes solubilized with 3–14 were a dimer and monomer, respectively (12). The results in this study of the inhibition by EEDQ substantiate this earlier hypothesis.

MATERIALS AND METHODS

Isolation of Plasma Membranes

Maize seedlings (*Zea mays* L. cv W7551) were germinated on filter paper moistened with 0.1 mM $CaCl_2$ (27). Microsomes were isolated from 3-d-old roots by differential centrifugation as described previously (8), except that the 25 mM bis-tris-propane (pH 7.8) in the homogenizing buffer was replaced with 50 mM Hepes (pH 7.5). Microsomes were suspended in 5 mL of homogenizing buffer and layered over a gradient consisting of two 10-mL steps of 34 and 42% (w/w) sucrose in 5 mM Hepes (pH 7.5). After centrifuging for 125 min at 40,000 rpm in a Ti 70 rotor at 4°C, the membranes collecting at the interface between the 34 and 42% (w/w) sucrose steps were removed, diluted 3- to 4-fold with 5 mM Hepes (pH 7.5), and then centrifuged at 55,000 rpm in a Ti 70 rotor for 65 min at 4°C. The final pellet was suspended in 20 mM Hepes (pH 7.5), 0.25 M sucrose, and 10% (w/v) glycerol at 5 mg protein/mL. Protein concentration was determined by a modification of the Lowry method after precipitation by TCA using BSA as a standard (6). Purification of plasma membranes resulted in a slightly greater than 3-fold increase in the specific activity of the vanadate-sensitive ATPase (data not shown).

Solubilization of Plasma Membrane Proteins and Fractionation by Size

The detergent 3–14 was dissolved at 40 mg/mL in the 20 mM Hepes buffer described above and sonicated for 5 min immediately before use. Plasma membranes were treated with 0.75 mg of 3–14 per mg of protein for 5 min on ice and then centrifuged at 55,000 rpm in a TLA 100.3 rotor for 60 min at 4°C. The resulting supernatant was used as the 3–14-solubilized plasma membrane protein. Where indicated, the supernatant was adjusted to 0.2% (w/v) DOC by the addition of 10% (w/v) DOC in water.

When the 3–14-solubilized protein was fractionated by glycerol gradient density centrifugation, the solution was diluted with an equal volume of 5 mM Hepes (pH 7.8). The diluted protein solution was layered over an 11-mL linear gradient of 15 to 40% (w/v) glycerol in 5 mM Hepes (pH 7.8) containing 4 mg/mL of 3–14 either in the presence or absence of 0.2% (w/v) DOC. After centrifugation for 16 to 18 h at 30,000 rpm in a SW 40 rotor at 4°C, the gradient was fractionated into 15-drop aliquots.

Plasma membrane proteins solubilized with 3–14 also were fractionated by HPLC using 600 × 7.5 mm Bio-Sil SEC-400[2] equilibrated with 50 mM Hepes (pH 7.8), 50 mM KNO_3, 5 mM NaN_3, and 4 mg/mL 3–14 either in the presence or absence of 0.2% (w/v) DOC. Two hundred microliters of protein were eluted with the equilibration buffer at a flow rate of 0.9 mL/min and fractionated into 0.9-mL aliquots. The approximate molecular masses of solubilized plasma membrane proteins were determined by the elution of cross-linked polymers of BSA.

Assays for Vanadate-Sensitive ATPase Activities

ATP hydrolysis by the corn root plasma membranes was monitored either by the release of inorganic phosphate or by following changes in NADH oxidation at 340 nm in the presence of lactate dehydrogenase, phospho*enol*pyruvate, and pyruvate kinase, as described previously (32). The assay media contained 17.5 mM Mes (titrated to pH 6.45 with bis-tris-propane), 0.2 mM EGTA, 2.5 mM $MgSO_4$, 2 mM ATP, and 50 mM KNO_3. Vanadate-sensitive ATPase activity was determined by measuring the difference between the rate of ATP hydrolysis in the absence and presence of 0.2 mM sodium *ortho*-vanadate. Throughout these investigations, plasma membranes exhibited 320 ± 20 nmol Pi min^{-1} mg^{-1} protein under standard assay conditions. Data are the average of at least two experiments of three to five replicates. Bars indicating the magnitude of the SE are included in figures when the variation exceeds the size of the data symbol. Proton transport was followed by changes in the absorbance of 7.5 μM acridine orange as described previously (10) in an assay buffer identical to that described above for ATP hydrolysis. The initial rate of proton transport was calculated as described previously (10).

The rate of ATP hydrolysis by the canine kidney (Na + K) ATPase (Sigma Chemical Co.) was also determined by the release of inorganic phosphate as described previously (10). The assay media contained 20 mM Hepes (pH 7.5), 100 mM NaCl, 25 mM KCl, 5 mM $MgSO_4$, 5 mM ATP, and 0.2 mM EGTA. The activity determined by measuring the rate of ATP hydrolysis in the absence and presence of 0.1 mM sodium *ortho*-vanadate was attributed to the (Na + K) ATPase, which averaged 0.7 μmol min^{-1} mg^{-1} protein under standard conditions.

EEDQ Treatment

Typically, 200-μL aliquots of either native or detergent-treated plasma membrane proteins were warmed to room

[1] Abbreviations: EEDQ, *N*-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroquinoline; 3–14, *N*-tetradecyl-*N*,*N*-dimethyl-3-ammonio-1-propane-sulfonate; DOC, deoxycholate.

[2] Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

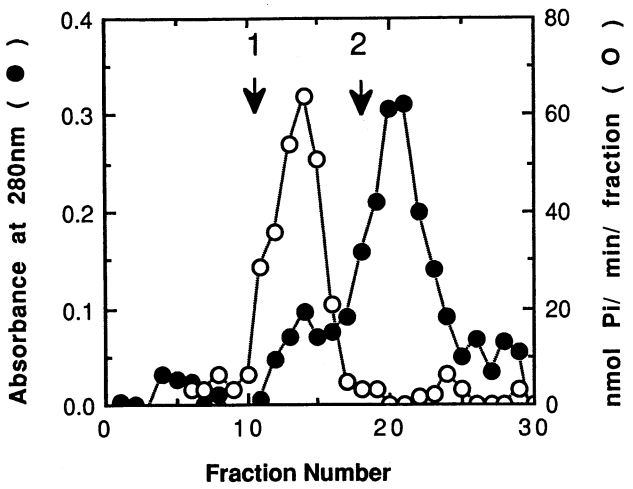

Figure 2. Distribution of 3-14-solubilized plasma membrane protein and vanadate-sensitive ATPase fractionated by size exclusion HPLC. Plasma membranes treated with 3-14 and the resulting solubilized portion were fractionated in the presence of 3-14 on a Bio-Sil SE-400 column as described in "Materials and Methods." Fractions were assayed for protein by absorbance at 280 nm (●) and for vanadate-sensitive ATPase activity (O). Arrows numbered 1 and 2 denote the volume at which BSA dimer and monomer eluted, respectively.

of 3 mM Mg increased from 0.26 mM for control vesicles to 0.48 mM for EEDQ-treated vesicles. These results suggested that EEDQ altered the ability of the ATPase to bind substrate. However, changes in activity induced by EEDQ treatment under standard assay conditions (i.e. 2.5 mM ATP) should reflect primarily alterations in maximum velocity because the ATP concentration is at least 5 times greater than the K_m.

The influence of ATP on EEDQ inhibition of the plasma membrane proton pump also was assessed. Plasma membranes were incubated with 2 mM EEDQ for 15 min at 18 to 22°C in the absence and presence of 1 mM $MgSO_4$ and 1 mM ATP. Vanadate-sensitive ATPase activity decreased by about 58% in the absence of Mg and ATP and by only 20% in the presence of 1 mM $MgSO_4$ and 1 mM ATP. Such a reduction in EEDQ-induced inhibition suggests either that the site of modification is near to the ATP binding location or that ATP binding induces a conformation change in the enzyme to shield the site of inhibition.

The initial rate of proton transport was more sensitive to inhibition by EEDQ than ATP hydrolysis. For example, a 10-min treatment of freshly isolated plasma membrane vesicles with 4 mM EEDQ resulted in decreases of approximately 70 and 30% for the initial rate of proton transport and vanadate-sensitive ATP hydrolysis, respectively (Table I). In this experiment, proton transport was totally abolished after 20 min of treatment, as compared to only 70% inhibition of ATP utilization by the pump. After 30 min, ATP hydrolysis had declined by almost 90%, whereas proton transport remained undetectable.

The ATPase was incubated in various concentrations of EEDQ for various periods of time to gain insight into the stoichiometry between EEDQ modification and the inhibition

Table I. *Effect of Incubation with 4 mM EEDQ on the Activities of the Plasma Membrane Proton Pump*

Incubation Time	ATP Hydrolysis	Initial Rate of H^+ Transport
min	*nmol Pi·min^{-1}·mg^{-1} protein*	*A·min^{-1}·mg^{-1} protein*
Control	310 ± 16[a]	0.21 ± 0.03[a]
0	300 ± 12	0.20 ± 0.02
10	229 ± 12	0.06 ± 0.02
20	87 ± 8	0
30	29 ± 6	0

[a] Average of two experiments each with three replicates ± SE.

of ATP hydrolysis. A straight line was obtained when the natural logarithm of the activity remaining was plotted as a function of incubation time at a particular EEDQ concentration (data not shown), indicative of a first-order reaction. A pseudo-first-order rate constant for inhibition and then a half-time for inhibition were calculated for each EEDQ concentration. The effective ratio between EEDQ and ATPase needed to produce inhibitory species was determined by the method of Levy et al. (26). A straight line with a slope of 0.51 was obtained when the logarithm of the reciprocal of the half-time of inhibition was plotted as a function of the logarithm of EEDQ concentration (Fig. 3). From the theory presented by Levy et al. (26), the slope of the log-log plot in Figure 3 is the minimum number of EEDQ moles necessary to inhibit 1 mol of ATPase, i.e. 1 mol of EEDQ inhibits 2 mol of ATPases. These results are consistent with conclusions from irradiation inactivation (12) that the functional structure

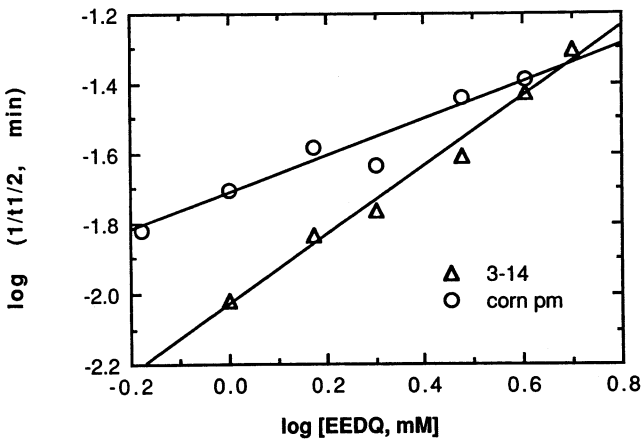

Figure 3. Inhibition of the plasma membrane vanadate-sensitive ATPase by EEDQ. The concentration and time dependence of EEDQ inhibition of the ATPase in plasma membrane (O) and 3-14-solubilized preparations (Δ) were analyzed by the method of Levy et al. (26). The pseudo-first-order rate constants for EEDQ inhibition were determined from the slopes of plots of the activity remaining as a function of time at a particular EEDQ concentration. Half-times for EEDQ inhibition were estimated from the pseudo-first-order rate constants, thus enabling the construction of the log-log plot between 1/half-time and the EEDQ concentration.

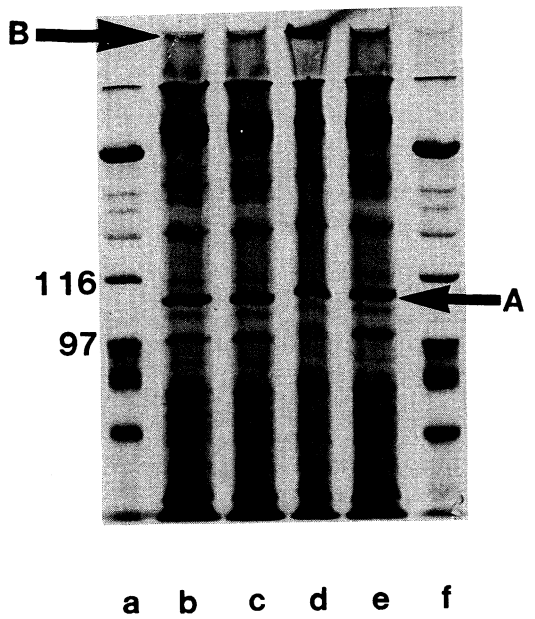

Figure 4. Polypeptide pattern of plasma membranes and 3–14-solubilized proteins treated with EEDQ. Plasma membranes or 3–14-solubilized proteins were treated for 60 min at 18 to 22°C with 2 mM EEDQ and immediately were subjected to SDS-PAGE as described in "Materials and Methods." Polypeptide profiles of untreated and EEDQ-treated plasma membranes appear in lanes b and d, respectively, whereas untreated and EEDQ-treated solubilized proteins appear in lanes c and e. Molecular mass standards of 116, 97, 68, and 42 kD appear in lanes a and f. Arrows A and B denote the location of a 100-kD polypeptide presumed to be that of the P-type ATPase and a high molecular mass aggregate, respectively.

of the ATPase in plasma membrane vesicles is a dimer of the 100-kD subunit.

The validity of the present approach was tested further by examining the effects of EEDQ on another membrane protein that is reported to be active as a dimer, the canine (Na + K) ATPase (24, 29). There was a time-dependent decrease in the activity of the (Na + K) ATPase when incubated in the presence of EEDQ (data not shown). Straight lines were obtained when the natural logarithm of the activity remaining was plotted as a function of time of EEDQ treatment (data not shown). The slope from the log-log plot of reciprocal of the half-time for inhibition as a function of EEDQ concentration was 0.58. This value is very close to the 0.5 predicted from previous studies, indicating that the (Na + K) ATPase functioned as a dimer (24, 29). A stoichiometry of one molecule of EEDQ inhibiting two molecules of ATPase observed in this report for the P-type ATPases from corn root plasma membranes and canine kidney tissue is in contrast to a stoichiometry of 1 to 1 found previously for the P-type ATPase from *Neurospora crassa* plasma membrane (1).

Based on the results from irradiation inactivation, solubilization of the plasma membrane ATPase by 3–14 reduces the structure to one 100-kD subunit per functional complex (12). Results from glycerol density centrifugation and HPLC presented earlier in this manuscript also indicated that the 3–14-solubilized ATPase was composed of one subunit (Figs. 1 and 2). Therefore, it was of interest to analyze the pattern of EEDQ inhibition with the 3–14-solubilized ATPase. When the inhibition data were analyzed according to the theory of Levy et al. (26), a slope of 0.99 was found for the log-log plot of the reciprocal of the half-time for inhibition as a function of the EEDQ concentration (Fig. 3), implying that 1 mol of EEDQ inhibited 1 mol of solubilized ATPase. Therefore, solubilization of the corn root P-type H^+-ATPase with 3–14 corresponded to an alteration in the characteristics of EEDQ inhibition that was consistent with a change in subunit composition.

One property of EEDQ is the activation of carboxylic acid groups in such a way as to catalyze the formation of peptide bonds between nearby carboxylic acids and amines (4, 5). Because of this property, EEDQ has been shown to induce the formation of high molecular mass aggregates in membranes by cross-linking adjacent polypeptides (1). The polypeptide profiles of plasma membranes and 3–14-solubilized proteins in untreated preparations were compared to those of preparations treated with 2 mM EEDQ for 60 min at 18 to 22°C (Fig. 4). There was consistently a greater amount of stained material remaining at the origin with EEDQ-treated membranes than with untreated membrane, solubilized protein, or EEDQ-treated solubilized proteins. The presence of the material at the origin with EEDQ-treated membranes is consistent with the formation of large molecular mass aggregates. These results support the hypothesis that the 100-kD polypeptides are closer to each other in membranes than in solubilized preparations.

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